

Genetic Variability among Three Species of *Aspergillus* 1. Seed born *Aspergillus* Sp. And Aflatoxins Associated with Dry Beans (*Phaseolus Vulgaris* L.)

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Abstract: *Aspergillus* sp. is one of the pathogenic plants attacked beans (*Phaseolus vulgaris* cv. Kontender) in storage and caused aflatoxins production. Five isolates belong *Aspergillus* include *A. niger*, *A. flavus*, *A. parasiticus*, *A. ochraceus* and *A. spp.* were isolated using agar plate (PDA) medium. The percentage of seedborne were 52.5, 0.75, 1.2, 4.5 and 5.1% respectively. It was observed differentiations of all morphologically growth between them on PDA medium. Conidial dimensions differed among *A. flavus*, *A. niger* and *A. parasiticus* was found to be 5.50 x 4.57; 3.75 x 1.53; 5.00 x 4.42; length/width ratio were 0.830, 0.674, 0.884 and volumes were 61.65; 23.90 and 53.90 μm^3 respectively. Nuclear and mitotic chromosomes of three species were observed with light microscope. The mean number of nuclei were 1.3; 1.4 and 1.5 respectively. The number of chromosomes were 16 for all 3 species. In *A. flavus* and *A. parasiticus* some chromosomes had a threadlike structure (TLS). Level of aflatoxins (B_1 , G_1 , B_2 and G_2) were determined with *A. niger* B_1 and B_2 (0.09 and 0.004) and *A. parasiticus* B_1 and G_1 (0.026 and 0.025) and *A. flavus*, B_1 , G_1 , B_2 and G_2 (23.5, 0.4; 0.34 and 0.20 ppm). Aflatoxins were not detected with other species of *Aspergillus*.

Key words: *Aspergillus* sp., Conidial dimensions, Aflatoxins, HPLC

INTRODUCTION

Aspergillus is a fungal genus including many species cause diseases in a wide range of plants. This genus is recognized by its distinct conidiospores terminated by a swollen vesicle bearing flask-shaped apical ends. The phialides may be borne directly of the vesicle or on intervening metulae. Some species may form masses of thick-walled cells called hülle cells. The spores come in several colours, depending upon the species and are produced in long chains from the ends the phialides. Commonly isolated from soil, plant debris and house dust, sometime pathogenic to man. To elucidate the nature of these species, a number of morphological, biochemical and phytopathological studies have been conducted. However, perhaps because of the small size of the vegetative hypha, few cytological studies have been done on the genetically and decay and mycotoxins production. Aflatoxins (B_1 , B_2 , G_1 and G_2) are produced by some strains of *Aspergillus flavus* and most, if not all, strains of *A. parasiticus*. These two species are ubiquitous storage fungi of seeds and grains, particularly peanuts, corn, beans and edible nuts, and they develop saprophytically on a wide variety of foodstuffs^[3]. The genus *Aspergillus* (10 species and 8 varieties) was the most common contamination by using PDA and blotter methods El-Shafie and Domijan *et al.*^[4,6]. Some isolates of *Aspergillus* were produced mycotoxins and *Aspergillus flavus* was produced aflatoxins B_1 , B_2 , G_1 and G_2 in infected legume seeds^[13,16].

The present study investigation was conducted to determine chromosome number, observe the nuclear number, dimensions of conidia and aflatoxins production of three species of *Aspergillus* that cause diseases of beans.

MATERIAL AND METHODS

Agar plate method was used for examine seeds of *Phaseolus vulgaris* cv. kontender collected from market. The seeds were disinfected by soaking in 5% sodium hypochloride solution for 2 min then washed several times with sterilized water and dried. This seeds were transferred onto potato dextrose agar (PDA) plates Neergard^[10], (5 seeds/dish and 10 dishes as replicates). All dishes were inoculated at $25^\circ \pm 1^\circ\text{C}$ under darkness for 5 days. The fungal growth was transferred and purified using hyphal tip techniques onto PDA medium contained streptomycin. The fungi were transferred to PDA slants. Pure fungal cultures were identified in the plant pathology Dept., Fac. of Agric., Ain Shams Univ. according to Gilman, Barnett and Hunter and Domsch *et al.*^[7,2,5]. Cultures were kept at 5°C for further studies.

Preparation of conidia: Single conidium of three *Aspergillus* isolates (*A. niger*, *A. parasiticus* and *A. flavus*) were grown on PDA medium. The isolates were incubated for 5 days at $25^\circ \pm 2^\circ\text{C}$ initially in the darkness, exposed 2 days to near ultraviolet light (FL205, B. Masushita, Osaka) and sporulated under dark conditions

for 2 more days. Conidia thus formed were collected and concentrated to 1×10^8 spores/ml in distilled water and stored at -80°C until use. Conidia suspended in distilled water were photographed with a light microscope (Microphor-FX, Nikon, Tokyo) fitted with an objective 40X (Plan A[p, 40 Nikon). The size 100 conidia in each isolate were measured using micrometric slide. The volumes of conidia were calculated as follows: Conidial volume (μm^3) = $L \cdot W^2 / 6$, in which L = conidial length (μm) and W = conidial width (μm).

Nuclei in conidia were observed as described previously^[14].

Observation of chromosomes: Conidia suspended in PDA were incubated on glass slides at $25^\circ \pm 2^\circ\text{C}$ for 20 hr and the germlings were treated with methanol-acetic acid solution (17:3 V/V) for 30 min and flame dried. The specimens were transferred to 95% ethanol and kept in 70% ethanol for 3 hr. After hydrolyzation in 1 N HCl for 5 min. at room temperature and 10 min at 60°C , they were washed with distilled water and stained with Giemsa (Giemsa's Losung, Merck, Darmstadt) at 3.5%, V/V, in 1% 1/1.5 M phosphate buffer, pH 7.0 for 3 hr. After being rinsed in tap water, they were air dried and observed with light microscope fitted with an objective lens (100X).

Extraction and quantification of aflatoxins: Aflatoxin were determined in Mycotoxins Central Lab. and Food Safety, National Research Center (NRC) according to^[1]. Each of *A. niger*, *A. parasiticus* and *A. flavus* isolates were grown in flasks (500 ml) containing 50 gm seed beans var kontender and enough moisture after autoclaved and incubated at $25 \pm 2^\circ$ for 15 days, then extracted and prepared to determine the aflatoxins using High Performance Liquid Chromatography System (HPLC). Twenty five ml media added to 100 ml chloroform then filtrate by sodium sulphate anhydrous (NaSO_4). Evaporation dry film at 40°C . Then added 10 μl (Trifluoroacetic acid (TFA) at room temperature for 15 min. then added 400 μl acetonitrile : water (2:18). 10 μl of the mixture was injected in HPLC.

Condition HPLC:

Mobile phase, H_2O : Methanol : Acetonitrile (6:3:1)

Flow rate 1.00 ml/min

Fluorescence detector. EX. 365 nm, Em. 450 nm.

Reverse phase C18 phenomenex, Luna 5 μ 250 x 4 nm

The data were integrated and recorded by Millennium Chromatography Manager Software 210 (Waters Milford MA 0775).

RESULTS AND DISCUSSIONS

Results: Agar plate method used for isolation of *Aspergillus* sp. from bean seeds. Results showed that disinfection of the tested seeds gave higher percentages of seed germination (92%). On the contrary the germination of infected seeds were lower (84%). The percentage of associated fungi was higher frequency and contaminated (77%) with seed samples belonging to 5 genus, *Alternaria*; *Aspergillus*; *Epicoccum*; *Fusarium* and *Trichoderma*. Five species of *Aspergillus* including *A. niger* was the most frequency and contaminated seeds with 52.5%, *A. flavus* was less frequency (6.75%) and *A. parasiticus* (1.2%), *A. ochraceus* (4.5%) and *A. spp.* (5.1%) moderate frequency.

Conidial morphological differed among the three species studies (*A. niger*, *A. parasiticus* and *A. flavus*). It was found, *A. flavus* has thickness layer of cell wall and large size of conidia spores than other two species. Conidial dimensions differed between three species (Table 1). The dimension of three species were 3.75 x 2.53; 5.00 x 4.42 and 9.50 x 4.57, the ratio of length/width were 0.674, 0.884, 0.830 and the volume were 23.90; 53.09 and 61.65 respectively.

Conidial dimensions of length and width may be used to distinguish *A. niger*, *A. parasiticus* and *A. flavus* from each other.

Microscopic observation of mitotic chromosomes in each species are shown in Table 1 and Fig. 1 are enlarged photographs of chromosomes of each species. All three species of *Aspergillus* had 16 chromosomes and *A. parasiticus* and *A. flavus* one and two chromosomes had a threadlike structure respectively.

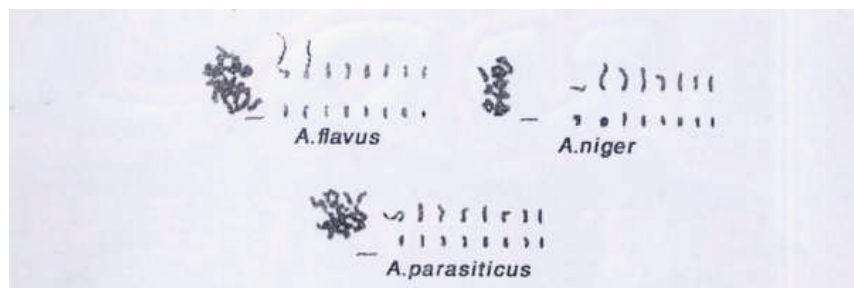


Fig. 1: Enlarged photographs of the chromosomes of *Aspergillus* species at metaphase. The chromosomes are arranged in descending order of length. Arrows indicate the threadlike structure Bar = 2 μm .

Table 1: Dimensions, number of nuclei and chromosomes of *Aspergillus* sp. conidia.

Species	Dimensions		Length/ width ratio	Volume (μm^3)	Chromosomes		No. of nuclei
	Length (μm)	Width (μm)			Number	TLC	
<i>A. niger</i>	3.75 \pm 1.1	2.53 \pm 0.5	0.674 \pm 0.3	23.90 \pm 23.6	16	0	1.3
<i>A. parasiticus</i>	5.00 \pm 1.7	4.42 \pm 1.0	0.884 \pm 0.1	53.08 \pm 189	16	1	1.4
<i>A. flavus</i>	5.50 \pm 1.7	4.57 \pm 1.1	0.830 \pm 0.2	61.65 \pm 200	16	2	1.5

Substrate PDA = potato dextrose agar medium.
TLC = Threadlike structure.

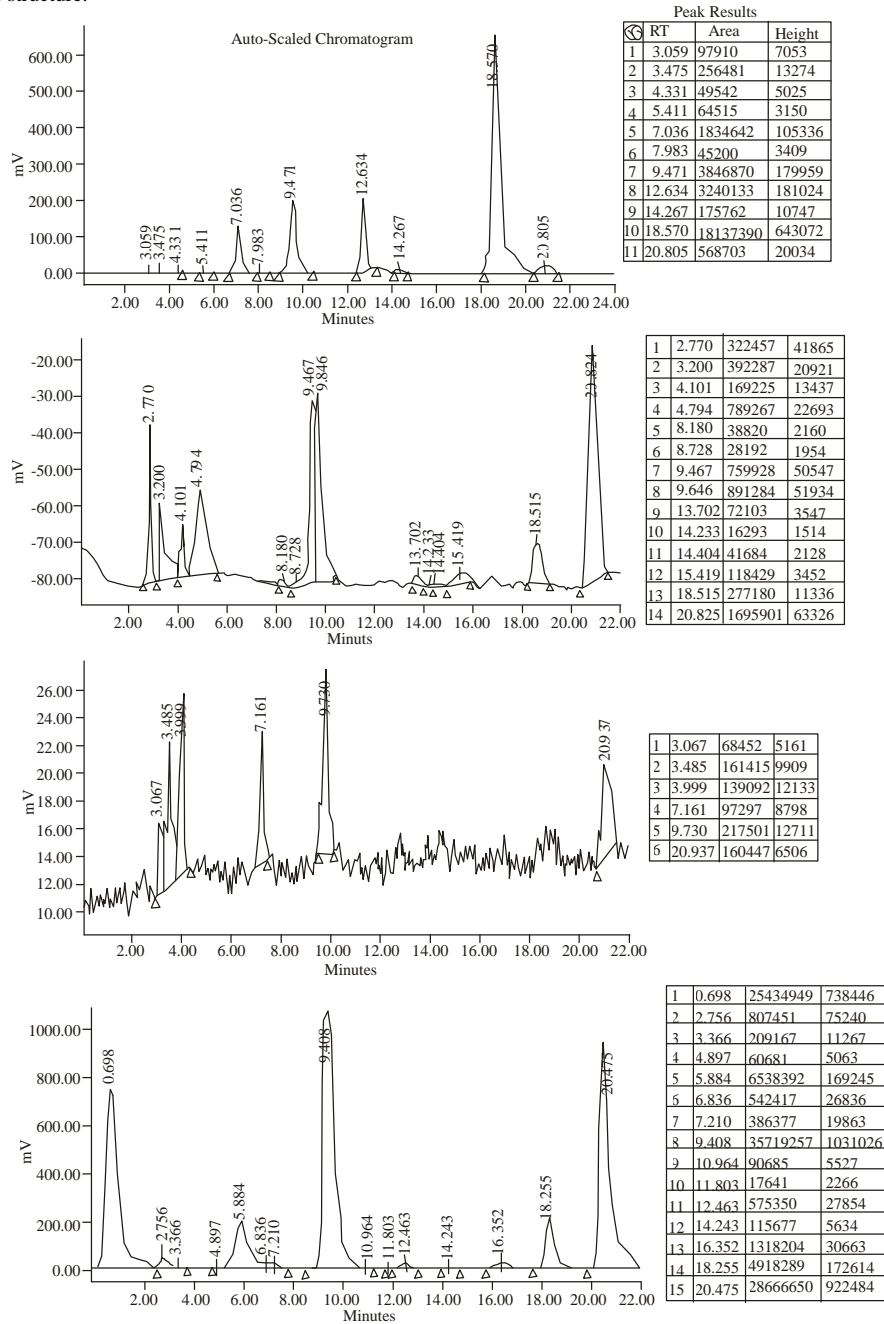


Fig. 2: HPLC quantification of aflatoxins (G_1, G_2, B_1, B_2) produced by *A. Flavus*, *parasitica* and *A. Niger* in PDA media O = Refer to the data of peak ranged in retention time range, * = Retention time of the standard aflatoxin ± 0.2 min

Table 2: Aflatoxins type produced by isolates of *Aspergillus* spp. in PDA medium ppm quantitative.

Aflatoxin type	<i>A. flavus</i>	<i>A. parasiticus</i>	<i>A. niger</i>
G ₁	0.40*	0.025	-
B ₁	23.5	0.026	0.09
G ₂	0.2	-	-
B ₂	0.34	-	0.003

* Quantitative ppm

- Non producing in medium.

Aflatoxins production: The extraction and quantification of Aflatoxins (B₁, B₂, G₁ and G₂) were determined using HPLC. Data were recorded in Table 2 and Fig. 2. Data show that isolates of *A. niger*, *A. parasiticus* and *A. flavus* were positive producer of Aflatoxins while other isolates were negative producer. Where, *A. flavus* gave the highest level of aflatoxins, G₁, B₁, G₂ and B₂ since gave 0.4, 23.5; 0.2 and 0.34 ppm/25 ml medium respectively, followed with *A. parasiticus* were gave G₁ and B₁ since gave 0.025 and 0.026 ppm/25 ml medium respectively, as well as *A. niger* where gave B₁ and B₂ since gave 0.090 and 0.003 ppm/25 ml medium respectively.

Discussion: Legumes is one of the most important plants family in Egypt, for local consumption and exportation. Several fungi attacked the legume plants during growth, at harvest and storage period. Isolation of seed-borne fungi which contaminated of bean seeds (*Phaseolus vulgaris* var. Kontender) yielded fungal isolates belong genera as *Alternaria*, *Aspergillus*, *Epicoccum*; *Fusarium* and *Trichoderma*. The most common seedborne fungi on dry beans were *A. niger* (52.5), *A. ochraceus* (4.5); *A. parasiticus* (1.2%), *A. flavus* (0.75) and *A. spp.* (5.1%). Similar results were obtained by El-Nagerabi and El-Shafie, Kritzinger *et al.*^[6,8,4] and Domijan *et al.* Disinfected seeds were enhanced and gave higher of germination percent and less fungal colonies compared with non disinfected seeds^[6,9].

Some isolates of *Aspergillus* were produced aflatoxins in dry bean seeds and PDA medium, *Aspergillus flavus* isolate was produced aflatoxins types G₁, B₁, G₂ and B₂; *A. parasiticus* was produced G₁ and B₁ and *A. niger* was produced B₁ and B₂ while other isolates of *Aspergillus* were not produced Aflatoxins. The same results were obtained by Tseny *et al.* and Ruiz *et al.*^[17,3].

Conidial dimension differed among three isolates of *Aspergillus* studies. The size of three isolates were 5.50 x 4.57, 3.75 x 2.53 and 5.00 x 4.92; ratio of length/width were 0.830, 0.674 and 0.884 and volumes were 61.65; 26.90 and 53.09 m³ for *A. flavus*; *A. niger* and *A. parasiticus* respectively. In this study, almost all size, volumes and length/width ratio of three isolates of *Aspergillus* full within the range described by Owen *et al.*^[11,15] and Shirane *et al.* as well the conidia are oblong to epiliptical in shape. The number of nuclei in conidia was observed similar for *A. flavus*, *A. parasiticus* and

A. niger. Phillips *et al.* and Shirane *et al.*^[12,15] reported that cultures of *Botrytis cinerea* growing on various media differ in the size and nuclear number of conidia. In the present study the substrate used to produce the conidia was not equal and could have influenced the results. The number of mitotic chromosomes in each isolate through 3 are enlarged photographs of chromosomes of each isolates. All three isolates of *Aspergillus* that were collected from dry bean seeds had 16 chromosomes. Therefore, the number reported previously for isolate S-v-5^[14] may be basic for this species. All isolates of *Aspergillus* had 16 chromosomes but *A. flavus* two and *A. parasiticus* one of the chromosomes has a threadlike, structure TLS and *A. niger* had not a threadlike structure. These findings indicate that the chromosomes number is correlated with the volume of the conidium and suggest that the isolates that have 16 chromosomes may be haploid only one or two chromosomes were associated with TLS. We have no explanation for this and also cannot deny the possibility of over lapping the faint TLS with chromosomes.

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